

DEVELOPING GENETIC MARKERS IN VETIVER (*CHRYSOPOGON ZIZANIOIDES*)

by

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Abstract

Vetiver (*Chrysopogon zizanioides*) is an introduced species that has the potential to become invasive in the U.S. This tropical grass is part of the tribe Andropogoneae (Poaceae), which contains some of most aggressive weeds, including *Imperata*, *Miscanthus*, and *Microstegium*. Vetiver is cultivated in the Americas, Australia, Africa, and Asia for the perfume industry as well as traditional medicine. More recently Vetiver cultivars have been developed for erosion control, due to its deep roots system and drought resistance. “Sunshine”, a sterile variety, has been introduced into the U.S. for the purpose of erosion control; however, there are no morphological characteristics (except sterile flowers) that differentiate the sterile Sunshine, from non-sterile varieties. Thus, there is a chance that non-sterile genotypes could enter the U.S. and possibly become invasive.

The purpose of this study is to develop genetic markers to differentiate between the sterile and non-sterile genotypes. Determining the difference between the varieties may enable cultivators to better control the introduction of new plant material into the U.S. To begin marker development, four million Illumina sequences were generated for Vetiver using genomic DNA from the Sunshine cultivar. Bioinformatic software MSAT commander identified 5083 sequences with microsatellite repeat regions; containing two to six base pair repeats in arrays of at least 10 to 20 units. A total of 46 primer pairs were developed for these microsatellite repeats and were screened against the USDA and Australian National Seed Bank Vetiver collections. Initial genotyping, resulted in a total of eight potential primer pairs that are being further examined. If these primer pairs properly differentiate and identify the “Sunshine” genotype from the other genotypes, then they can be implemented as genetic markers for this species.

Introduction

Chrysopogon zizanioides L. Nash (Poaceae), commonly known as Vetiver, is a large perennial grass that has traditionally been used in religious ceremonies and the perfume industry, but has recently gained significance for erosion control and phytoremediation efforts (Vietmeyer, 1993; Danh, et al., 2009; Pareek and Kumar, 2013; Balhassen, et al., 2014). A sterile (non-reproductive) variety, known by the name “Sunshine”, has been introduced to the U.S. for the purpose of erosion control (Joy, 2009). However, the grass tribe Andropogoneae in which Vetiver belongs is known for producing some of our most noxious weeds and there are no morphological differences between the sterile and non-sterile varieties of Vetiver. There is some concern that non-sterile varieties could accidentally be imported to the United States, initiating the release of an aggressive non-native grass. The origin of the sterile variety is unknown; though it is thought to have originated in India or Indonesia because the oldest cultivars are found in this region (Adams, et al. 2008). The goal of this project is to develop microsatellite markers to identify the sterile variety from all others in hopes of providing a tool to facilitate the identification of non-sterile varieties of Vetiver.

Vetiver grows to an above ground height of about 2 meters with an inflorescence that is much higher containing thousands of florets (flowers) arranged along the top 15 cm to 45 cm of the stalk (Pareek and Kumar, 2013) (Figure 1 and 2). The leaves are typically 1 cm wide and can have sharp edges (Vietmeyer, 1993). Vetiver is widely known as a xerophyte, because of its deep root system that has been measured up to 4.6 meters deep. (Banerjee, et al. 2016) Many grass species perish if their crown, the parts of the plant above ground, is

covered in soil. Vetiver is unique in its ability to reposition the crown above ground (Vietmeyer, 1993). Vetiver can survive in a wide range of temperatures (-15 to 55 °C) with soil pH levels ranging from 3.3 to 9.5 (Danh, et al., 2009). Vetiver is also adapted to withstand low water availability (200 mm/yr), as well as high rainfall (2000 mm/yr) (Vietmeyer, 1993). Due to the extreme environments of India, Africa, and Australia where the genus *Chrysopogon* is native, Vetiver can be difficult to manage using fire, drought, or grazing (Vietmeyer, 1993). The only control methods that seem to work include destruction of the crown (apical meristem) or using glyphosate herbicides (Round-up) (Vietmeyer, 1993; Joy, 2009). The genus *Chrysopogon* has a base chromosome number of 10 and there are many polyploidy forms known (Lavania, 1988). Vetiver is normally diploid ($2N = 20$), but there are tetraploids ($4N = 40$) that were created to increase Vetiver oil production (Lavania, 1988; Dhawan and Lavania, 1996).



Figure 1. Vetiver on capitol grounds in Baton Rouge.



Figure 2. Vetiver Inflorescence.

Vetiver oil has been cultivated for over a century in most parts of Asia (Adams, et al. 2008; Balhassen, et al. 2014). The largest producers of Vetiver are Haiti, India, Java, and Reunion, which they export as a cash crop to India. India consumes the majority of Vetiver for traditional medicine (Yaseen, et al., 2014). Vetiver is also commonly used in the perfume industry as a major ingredient in many perfumes, lotions, and aftershaves (Balhassen, et al. 2014). The essential oils of Vetiver are extracted from the roots by a process known as hydrodistillation. Hydrodistillation, a form of steam distillation, involves soaking the Vetiver roots in water. The solution is then heated in a simple distillation set-up, and the steam is condensed and separated from the oil. This oil has a subtle scent of wood with other earthy

undertones. These undertones are widely dependent on geographical location of the grass, similar to wine grapes (Balhassen, et al., 2014). There is some debate if oil composition is solely dependent on genetics rather than geographic location or soil composition (Adams, et al., 2008). In some locations Vetiver oil is believed to have medicinal properties (Pareek and Kumar, 2013). In India, they commonly include Vetiver in traditional medicines to remedy a number of ailments including headaches, toothaches, skin disorders, and depression (Pareek and Kumar, 2013). There has been some experimental evidence that Vetiver oil may have some medicinal purposes such as an antibiotic or for anxiety relief, but much work must be conducted to verify these findings (Nirwane, et al., 2014).

Vetiver has recently been adopted for bioremediation efforts (Danh, et al., 2009; Banerjee, et al. 2016). Vetiver is frequently used to remove harmful chemicals from soil because it is tolerant of different regions and soil types (Sengupta, et al., 2016). Vetiver has been shown to absorb and utilize tetracycline in amino acid metabolism. Tetracycline is an antibiotic that is commonly used for bacterial infections in humans and is not well regulated outside of the developed world. Due to its excessive consumption, it's found in many parts of the environment in surface water, groundwater, and soil (Sengupta, et al., 2016). Studies show Vetiver could be used to remove tetracycline from soil, decreasing risk of the development of antibiotic resistance in bacteria (Sengupta, et al., 2016). Vetiver also acts as a bioremediator of heavy metals like lead, zinc, arsenic, and cadmium (Chiu, et al., 2006; Datta, Quisoe, and Sarkar, 2011). Vetiver can metabolize and degrade the heavy metals, making phytoremediation preferable over other methods of remediation (Ng, et al., 2016). Heavy metals do not cause damage to the nuclear DNA of Vetiver, suggesting that Vetiver could survive a long time as a phytoremediator (Danh, et al., 2009; Banerjee, et al. 2016).

Vetiver can withstand up to 225 mg/kg of arsenic, 300 mg/kg of zinc, 125 mg/kg of copper, and 361 mg/kg of lead (Datta, et al., 2011; Chiu, et al. 2006). In many cases, Vetiver is a cheaper option than the other bioremediation techniques including land filling, washing, bioleaching, and excavation of soil contaminants (Ng, et al., 2016). These expensive methods tend to be more destructive to the environment than phytoremediation (Banerjee, et al., 2016).

There are many different types of Vetiver. The majority of genetic diversity of Vetiver can be found in India (Singh, et al., 2014). *Chrysopogon zizanioides* is most closely related to *Chrysopogon gryllus* its sister species (Barnard, et al., 2013). It is believed that every cultivar outside of Southeast Asia is the Sunshine variety (Adams, et al., 2003). The sterile variety of Vetiver called Sunshine, appears to be related to “Zomba”, a variety commonly cultivated in Malawi (Dong, et al., 2003). The Sunshine variety can only produce asexually via propagation; it does not produce seeds (Celestino, et al., 2015) The United States Department of Agriculture has allowed the “Sunshine” variety to enter the US for the purpose of erosion control (USDA, 2016). Currently we do not understanding why this variety is sterile, but observations in Hawaii over many years demonstrate its inability to reproduce by seed (Joy, 2009). Other than the lack of seed set, there are no morphological differences between the sterile and non-sterile variety making it difficult to identify different varieties. There is a possibility that a non-sterile variety could enter the U.S. unintentionally allowing the uncontrollably spread as an invasive weed. A better understanding of why this plant is sterile might aid in preventing and detecting a noxious weed invasion.

Vetiver is a member of the genus *Chrysopogon*, which belongs in the grass family Poaceae, Panicoideae subfamily, and the tribe Andropogoneae (Clayton and Renvoize,

1986). Many of the grasses in this tribe are dominant in their native habitats and some have become invasive in non-native habitats (Clayton and Renvoize, 1986). Well known examples include Giant Miscanthus (*Miscanthus giganteus*) (Greif et Deu ex Hodgkinson et Renvoize), Johnson-grass (*Sorghum halepense*)(L. Pers.), and Congon-grass (*Imperata cylindrical*)(L. Beauv.). Not only do these species share common lineage with Vetiver, but they also share similar traits. Some of these traits include deep root system and drought resistance. Also, similar to the Sunshine variety, they use asexual propagation as their main source of reproduction. Sharing lineage and traits with these invasive grasses may indicate that Vetiver could have an increased risk of invasiveness.

Giant Miscanthus (*Miscanthus giganteus*) is native to Japan and is commonly grown on sub-optimal soils as a biofuel in much of Europe (Stewart, et al., 2009). The issue is that many grasses used for biofuels have the same qualities of invasiveness, including deep root systems and drought resistance. These two traits can also be found in Vetiver. The main mode of reproduction for *M. giganteus* is propagation, since the variety allowed into the U.S. is functionally sterile. The Weed Risk Assessment (WRA) was mostly responsible for assessing *M. giganteus* potential for invasiveness and creating models for vitality rates in regions of production. However, *M. giganteus* typically exceeds the population growth rate and region of growth, making it increasingly invasive. Indicating that the WRA was not effective in managing Giant Miscanthus populations (Matlaga and Davis, 2013).

Johnson-grass (*Sorghum halepense*) is another invasive tropical grass species found on farmland across the United States (Warwick, 1990). Like Vetiver, Johnson grass is thought to have originated in Asia and can grow up to two meters in height and compete with crop species like Maize or grain Sorghum. Johnson-grass has also been shown to use

allelopathy, chemical inhibition, to take over regions where native grasses were present (Rout and Chrzanowaski, 2009).

Cogon-grass (*Imperata cylindrica*) is also an invasive weed reducing quality fodder across the southern portions of the United States (Holzmueller and Jose, 2011). Cogon-grass is thought to have originated in Southeast Asia (USDA, 2006). The largest mode of reproduction is cloning; however, it is considered an out-crossing species. Its hearty rhizomes help perpetuate its survival. Because of cogon-grass's geographical range, it has easily colonized specific regions of the U.S. (Burrell, et al., 2015). This clonal reproduction is similar to the Sunshine variety of Vetiver. Cogon-grass has the ability to survive mowing and burning, similar to Vetiver. Genetic markers can be utilized to identify the locations of populations of cogon-grass in the U.S. These assist in assessing the growth of the populations as well as determine expansion of cogon-grass's geographical range (Burrell, et al., 2015).

There are three main attributes that lead to a species becoming invasive, recombination, hybridization, and naturalization. Recombination and high genetic diversity can allow the species to adapt to the introduced environment. Hybridization with related species that are already found in the region can make it easier for a plant to become invasive, by assimilating locally adapted alleles. Lastly, the easiest way is the plant being pre-adapted to the region. This means that they do not have to do anything to adapt to the environment (Burrell, et al., 2015). Vetiver is well adapted to the regions of the U.S. where they are being introduced. This is due to its ability to survive wide ranges in temperature, soil pH, and rainfall.

The main ways to regulate invasive species are to stop introduction into the environment, detect foreign species that have been naturalized, and by limiting the spread of

species invasion. Detection and controlling an invasive species is very expensive and key to preventing further invasion and destruction of habitats. However, prevention is the most successful and cheapest strategy in stopping an invasive species from entering the U.S. (Mehta, et al., 2007). The US Department of Agriculture (Aphis) is responsible for monitoring all foreign species entering the U.S. They aim to prevent invasive species from entering the country. Aphis inspection of cargo is the main form of protection against invasive species. Once non-native species are identified in a shipment, they can be destroyed or returned to the country of origin. However it's unlikely that the foreign species will be identified unless they are in high quantities (Mehta, et al. 2007). The USDA uses a Weed Risk Assessment to determine if a species should be allowed to enter the U.S. and determine if it can be classified as invasive. The weed risk assessment starts by assessing the ability for the taxon to spread and its invasive potential. Then, the assessment determines what impact it could have on the habitat it is entering. Finally, they determine which regions are suitable for establishing that species (USDA, 2016). In order to prevent the non-sterile varieties of Vetiver from entering the U.S., there has to be a way to determine the difference between the Sunshine variety and the other forms.

A good way to distinguish individuals or species is through microsatellite repeats. In Cogon-grass, markers were developed based on these repeats. These markers were then utilized for not only determining the species, but also for detecting the spread of Cogon-grass (Burrell, et al. 2015). Using microsatellite markers, such as those for Cogon-grass, are a fast and cost effective way to determine species and monitor them (Selkoe and Toonen, 2006). Microsatellite repeats are highly variable small repetitive regions in the genome of all eukaryotes that can be used to distinguishing differences between individuals. These

sequences consist of two to six base pair motifs (examples AT, TGC, GATT, etc.) that are arrayed in large blocks sometimes spanning 2000 base pairs or more. Variation in the array number of microsatellite repeats are normally caused by replication slippage, where DNA polymerase temporarily falls off the strand during replication (Viguera, et al., 2001)(Figure 3). When DNA polymerase reattaches it occasionally rereads a portion of what was already copied or misses a section of the array (Viguera, et al., 2001). In this way, arrays can expand or retract anytime the DNA is replicated. Microsatellites can occur in non-coding and coding DNA, though non-coding is more predominate. Microsatellite markers (PCR primers) are generally conserved within a species, but can also be shared by closely related taxa. Primers can be developed to amplify these regions to determine the array length (allele) of individuals and to examine if they are homozygous (same allele) or heterozygous at that locus. By combining data from multiple microsatellite regions, unique individuals can be identified (Selkoe and Toonen, 2006). The goal of this project is to develop microsatellite markers that would help identify the Sunshine variety in comparison to the other varieties of Vetiver. This would enable proper identification of the Vetiver that enters the U.S. and enable tracking of future Vetiver growth.

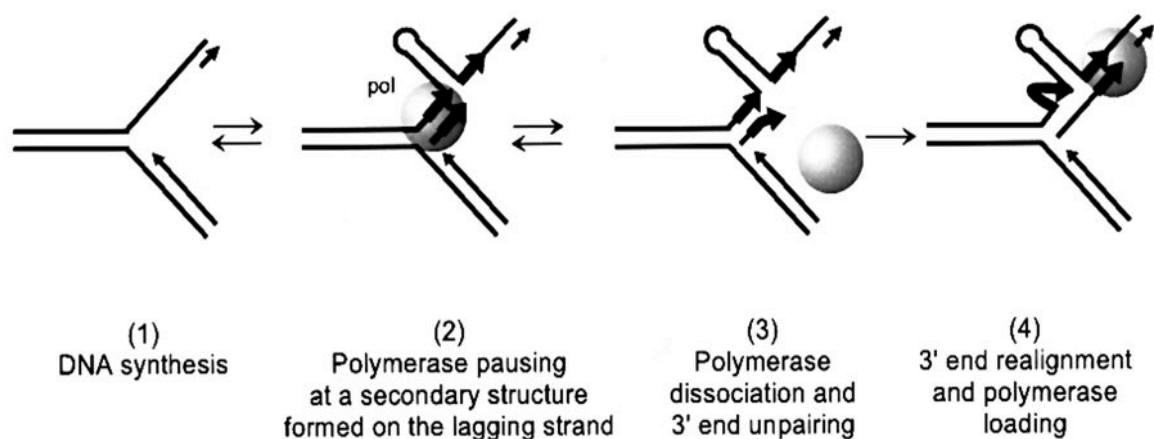


Figure 3. Replication slippage that forms microsatellite repeats. (Viguera, et al., 2001)

Methods

A collection of *Chrysopogon* species, from a variety of sources, was grown from seed or stabs (asexual reproduction) in the ASU greenhouse. This collection included 12 *C. zizanioides* individuals, seven unidentified *Chrysopogon* individuals, one *C. serrulatus*, and one *C. gryllus*. *C. serrulatus* and *C. gryllus* are closely related to *C. zizanioides*, so they were used for comparison. Among the *C. zizanioides* species there were two Sunshine individuals. There were also four biological replicates: PI 537061 had three replicates, PI 536999 had two replicates, PI 538757 had two replicates, and the two Sunshine (PI 671958) were replicates. The USDA seed bank collected the majority of the seeds, with a few coming from the Australian seed bank (Table 1). Most of the seeds from seed-banks originated in India.

Leaf tissue was collected from 21 *Chrysopogon* taxa and ground in liquid nitrogen. Genomic DNA was extracted from the individuals using a 2 % CTAB, cationic detergent used to break down polysaccharides, extraction (Doyle and Doyle, 1987). To increase DNA purity a 0.1 mg/mL concentration of Protease K (degrade proteins) and a concentration of 1 µg/mL of RNase (degrade RNA) were added to the extraction buffer before incubation at 65 °C for 24 hours. An organic extraction with Phenol:Chloroform:Isoamyl (25:24:1) was used twice to remove proteins and secondary metabolites. The samples were then precipitated with equal volumes of isopropanol and washed twice with 70 % ethanol to remove excess salt. Having pure DNA is important because proteins and RNA can alter the results of the PCR and primer annealing. CTAB extractions result in high DNA concentrations with low protein and RNA contamination. Each sample was quality and concentration checked by gel electrophoresis (Figure 4) and absorbance spectrophotometry (Nanodrop-1000). The DNA samples' qualitative characteristics were thick, even bands with little remaining material in the well (protein contamination) or signs of degradation. The qualifications for

spectrophotometry were a 260:280 ration around 1.7 – 1.9 and a 260:230 ratio close to 2.00.

All samples were diluted to a concentration of 20 ng/uL for downstream applications.

Table 1.

Chrysopogon individuals that were obtained from the USDA and Australian Seed Banks. This is the complete list of individuals used for testing the primers developed from MSAT commander.

Species	Seed Bank Identity	DNA ID	Origin
<i>C. zizanioides</i> (Capitol)	Q 37736	10	US
<i>C. zizanioides</i> (Sunshine)	PI 671958A	S	US
<i>C. zizanioides</i> (Sunshine)	PI 671958B	8	US
<i>C. zizanioides</i>	Angola	A	Angola
<i>C. zizanioides</i>	PI 504807	1	India
<i>C. zizanioides</i>	PI 538757A	5	India
<i>C. zizanioides</i>	PI 538757B	4	India
<i>C. zizanioides</i>	PI 196257	6	India
<i>C. zizanioides</i>	PI 537061A	7	India
<i>C. zizanioides</i>	PI 537061B	2	India
<i>C. zizanioides</i>	PI 537061C	9	India
<i>C. zizanioides</i>	PI 271633	13	India
<i>C. Sp.</i>	PI 536999A	12	India
<i>C. Sp.</i>	PI 536999B	19	India
<i>C. Sp.</i>	PI 504808	14	India
<i>C. Sp.</i>	AusTRCF106187	15	unknown
<i>C. Sp.</i>	PI 504802	16	India
<i>C. Sp.</i>	AusTRCF52213	17	unknown
<i>C. Sp.</i>	PI 504803	18	unknown
<i>C. Serrulatus</i>	PI 219580	3	Pakistan
<i>C. Gryllus</i>	PI 383762	11	Turkey

Illumina sequence data, generated by the lab PI, was used to identify microsatellite loci (per communication, Estep). These raw sequences were trimmed for low quality regions and the software MSATcommander was used to identify microsatellites regions within the sequences (Faircloth, 2008). Qualifications to be considered a microsatellite were repeat units consisting of di- to hexa-nucleotide repeats in arrays of at least 10 to 20 units. The software package also identified primer pairs for each microsatellite, requiring the annealing temperature (T_m) to be between 58 °C and 62 °C with an optimal temperature of 60 °C; a

G/C content ranging from 40 – 60 % with an optimal of 50 %; and that the forward and reverse primer T_m's were no more than 2 °C degrees different. A group of 46 high quality primers were finally chosen from the large group of 488 by identifying varying repeat motifs. Each of the forward primers were tagged on the 5' end using the M13 sequence (5'-CACGACGTTGTAAAACGAC-3') to facilitate fluorescent labeling of PCR products and downstream genotyping (Schuelke, 2000).

The primer pairs were amplified and screened using gel electrophoresis. The purpose of this step was to determine if the primer amplified a product or not. Each primer was tested against seven *Chrysopogon* individuals and a deionized water only negative control (Table 2). The seven *Chrysopogon* individuals were selected based on varying the types of individuals and not including any biological replicates. These were not screened against all of the *Chrysopogon* individuals because this portion was used to assure that the primer did amplify some portion of the DNA. The samples were amplified with each primer set using Polymerase Chain Reaction (PCR) on a Master Cycler thermocycler (Eppendorf Mastercycler). The reagents were used for PCR were the Promega GoTaq Flexi DNA polymerase kit. Each PCR reaction contained 0.41M of deionized water, 5X buffer (Ref M8295), 2.5mM MgCl₂, 0.2 mM dNTPs, 1 mM forward primer, 1 mM reverse primer, 1.25 U of Taq, 1µ of 20 ng/µL DNA, and deionized water to final 10µL volume. The thermocycler conditions for this were an initial denaturation of 94 °C for five minutes, followed by 13 cycles of touchdown. The touchdown includes 94 °C for 45 seconds, variable temperature between 68 °C and 55 °C for a two minute annealing, decreasing by 1° every cycle, followed by a 72 °C. extension Then there were 25 cycles of 94 °C for 45 seconds, 50 °C for one minute, and an extension of 72 °C for one minute. The final extension was for ten minutes at

72 °C. The variable annealing temperature was to improve the specificity of amplifications produced for each primer. The samples were then analyzed on a 1 % agarose gel and examined for the presence or absence of a PCR product. The primers were assigned a score based on the number of samples amplified (1-7). For example, a primer that amplified for all samples would receive a score of seven, while a primer that amplified for none of the samples would be scored as a zero.

Table 2.

Chrysopogon individuals selected for gel electrophoresis screening.

Species	Seed Bank Identity
<i>C. zizanioides</i> (Sunshine)	PI 671958A
<i>C. serrulatus</i>	PI 219580
<i>C. zizanioides</i>	PI 538757B
<i>C. zizanioides</i>	PI 537061A
<i>C. zizanioides</i> (Capitol)	Q 37736
<i>C. zizanioides</i>	PI 271633
<i>C. Sp.</i>	AusTRCF106187

The primers that passed the first screening were then genotyped on a 3730 sequencer (University of Georgia, Georgia genomics facility). Eight *Chrysopogon* individuals, out of the 21 individuals (Table 3) were amplified using the selected 40 primers and the thermocycler conditions described above. These primers were fluorescently tagged using FAM, VIC, PET, or NED, by adding a third primer, M13 (5'-CACGACGTTGTAAAACGAC-3') that was 5' labeled with one of the fluorophores. These products were multiplexed by dye set and sent for separation. The resulting chromatograms were scored based on presence of peaks for each sample. The size variation identified in the genotype data (amplicon) needed to match the targeted motif of the microsatellite it was amplifying. Each microsatellite marker was accessed for repeatability across the eight individuals. These scores were based on an A, B, C, D, F scale, with an A representing

amplifying for all 8 *Chrysopogon* individuals, B representing amplifying for all but one of the individuals, and F represented amplifying none of the individuals. Those primer pairs that did not score at the B level or above were consistently discarded.

Table 3.

List of samples used for first genotype screening.

Species	Seed Bank Identity
<i>C. zizanioides</i> (Sunshine)	PI 671958A
<i>C. zizanioides</i>	PI 504807
<i>C. serrulatus</i>	PI 219580
<i>C. zizanioides</i>	PI 538757B
<i>C. zizanioides</i>	PI 537061A
<i>C. zizanioides</i> (Capitol)	Q 37736
<i>C. Gryllus</i>	PI 383762
<i>C. zizanioides</i>	PI 271633

A second round of genotyping (third amplification test) was conducted to finalize testing of the primers. These 10 primers were screened against all 21 *Chrysopogon* individuals, using the same methods as described above. Data from these individuals allowed us to assess variability within each marker and to identify the range of amplicon sizes.

Results

DNA extractions from Vetiver resulted in gels that matched the qualities listed in the methods (Figure 4). There was little protein or RNA contamination and the concentrations of DNA were large (200 – 600 ng/uL) (Figure 5). The genome size of *Chrysopogon zizanioides* is approximately 1058 Mbp/1C. Since this was whole genome DNA extraction, it was expected that the bands in the gel would be larger than the molecular weight marker (1 kbp).

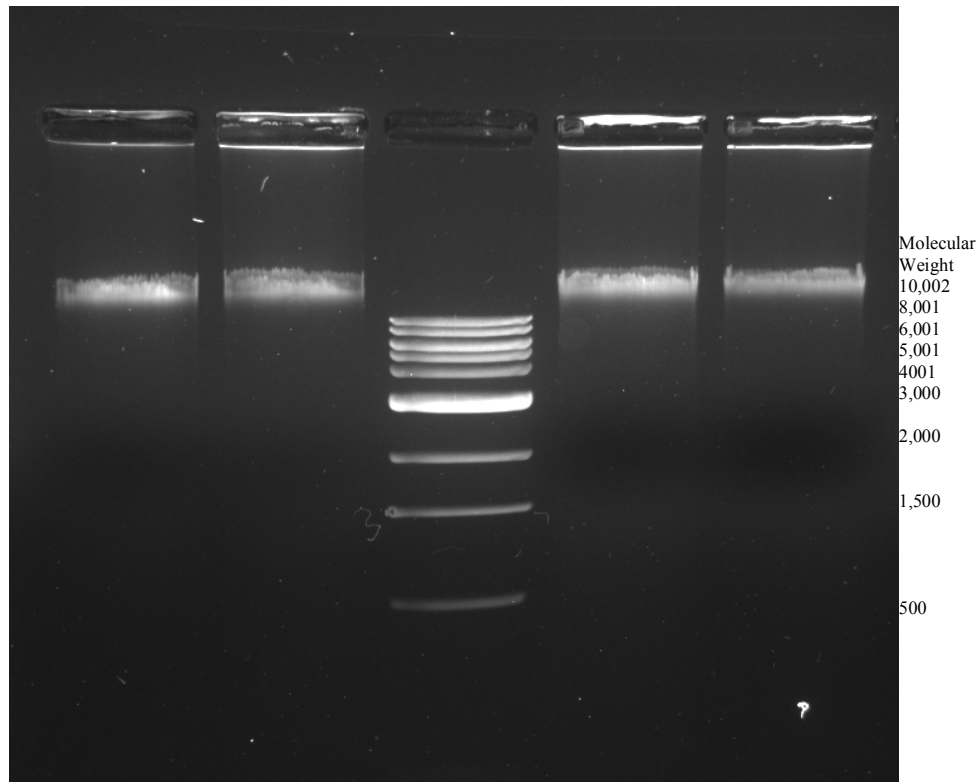


Figure 4. Example of DNA extraction imaged using ethidium bromide and UV light. From left to right: PI 219580, PI 538757B, 1 kb Ladder, PI 538757, and PI 196257. This shows extracted Vetiver DNA, with no DNA remaining in the well and thick even bands. These results show the quality of DNA that was ideal for testing each primer pair.

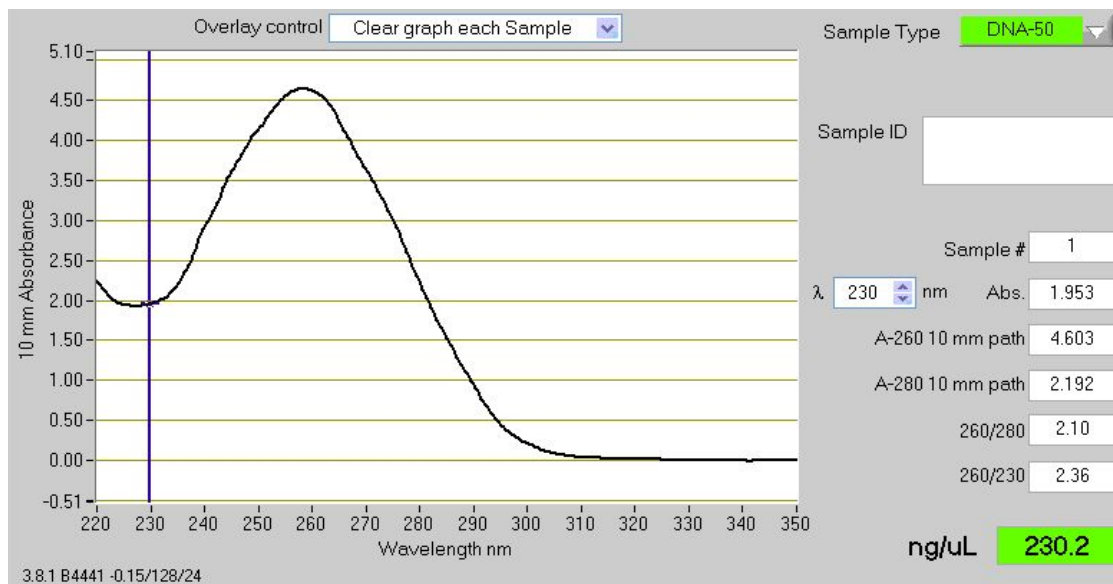


Figure 5. Absorbance Spectrophotometry of PI 219580. This is an example of the quality DNA for the spectrophotometry.

Illumina sequencing was able to generate 9,037,374 sequences (Figure 6). MSATcommander identified 5083 microsatellites that fit our qualifications with these sequences (see methods). A total of 488 primers were developed from these microsatellite regions. 46 primers met the qualifications listed in the methods. The primers were named CZ and numbered 1 – 46. For the first round of selection, all 46 primer pairs were amplified and products were assessed for the presence or absence of a PCR product on a 1 % agarose gel against seven *Chrysopogon* individuals (Table 1). Six primers failed to amplify a PCR product of the seven individual targets tested. These included CZ2, CZ20, CZ21, CZ45, and CZ46. Forty primer pairs were chosen for further analysis via gel electrophoresis (Figure 7). During the second round of selection, products were fluorescently labeled for genotyping. These primers were scored against eight *Chrysopogon* individuals (Table 2). Multiple fluorescent labels were used to determine different primers amplification of the same individuals. A total of 30 primers were eliminated because they did not amplify consistently across the majority of individuals, and had a score of C or less on the A – F scale. Ten primers were selected for a final round of genotyping based on their amplification across most of the *Chrysopogon* individuals (Table 4). The numbers of alleles per locus ranged from 5-7 with an average of 5.2. Marker CZ33 had the most alleles, seven alleles, while marker CZ5 had the fewest alleles, three alleles. There were no alleles identified that were specific to the Sunshine variety or the other sterile varieties.

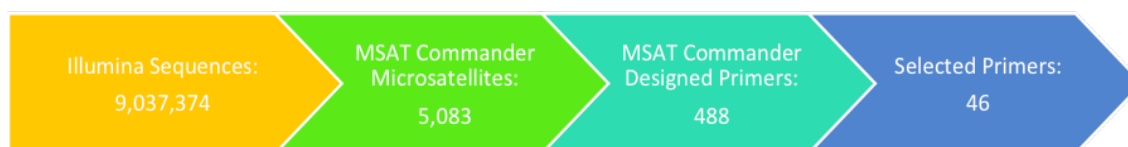


Figure 6. Primer development flow chart.

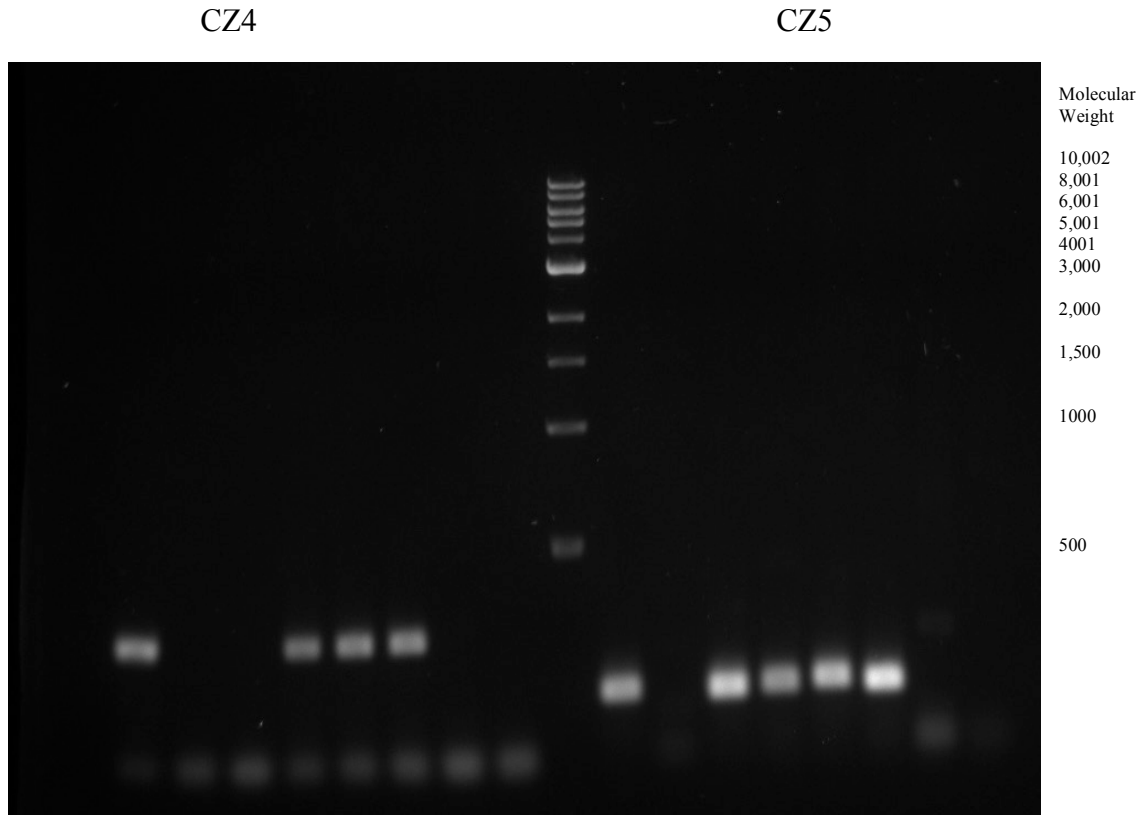


Figure 7. Example of gel electrophoresis scoring of primers imaged using ethidium bromide and UV light. CZ4 and CZ5 were scored based on the presence of a PCR product for the seven *Chrysopogon* individuals mentioned in Table 2. CZ4 would receive a score of four because it amplified for four *Chrysopogon* individuals, and CZ5 would receive a score of five because it amplified for five individuals. This image represents how each primer pair was scored based on the number of PCR products it produced during the first screening process.

The second genotyping showed that the ten microsatellite markers developed here (Table 4) were less likely to amplify in *C. serrulatus* and *C. gryllus*; however were consistent within the *C. zizanioides* collection. In addition, marker CZ13 had distinguishable peaks for four *Chrysopogon* individuals, two of which were Sunshine (Figure 8). The other two were PI 196257 and Angola. Each of these individuals have peaks that fit in the motif range of three nucleotides. It is expected that this would occur because CZ13 has a motif that is three nucleotides long. This shows that there is a possible genetic relationship between these individuals because they share a similar microsatellite.

Table 4.

Primers selected based on gel electrophoresis scoring and genotyping. Gel score and genotyping score indicate quality of marker. The ten primers listed were selected based on their gel score and the score developed by the first round of genotyping. These individuals were genotyped a second round for comparing quality of peaks.

Marker	Motif	Primer Sequence (5'-3')	Tm	Gel Score	Fluorescent Dye	Genotyping Score	Number of Alleles	Allele Size Length
CZ 1	(AAAG) ₇	F - TCCTTGCTCGATTCTCCTGG R - TTAGTGGTAGGTGGTGTGCC	60	6	PET	B	6	289-309
CZ 3	(AAAG) ₁₁	F - AAGCCTTCGAGCAAATGAC R - TTGGACCTTCAA8ATGGCCC	59.1	5	NED	B	6	247-277
CZ 5	(AAAT) ₆	F - TTAATCCATGCTACCACTGC R - TATGGACCACTATAGCGGCC	58.8	5	VIC	B	3	163-171
CZ 11	(AAG) ₉	F - AAGCTTCCAATTGCAAACTG R - TGCTATATGGAACCC TTCATGC	59	7	FAM	A	5	96-117
CZ 13	(AAT) ₁₁	F - GTTGCAGCTTTCAGGGACAG R - AACTAAGACGGCAGCTTTGG	59.7	7	VIC	A	5	163-196
CZ 22	(ACT) ₈	F - CTAATACATGGTGGGCATGC R - GCTGGATGGAGTAGTAAACGAC	59.2	7	NED	A	6	62-207
CZ 32	(AGC) ₈	F - ATTTGTTGTCTGGGATGCTCG R - CATGATGGTAAACAGGTGGGC	59.8	6	NED	A	4	215-224
CZ 33	(AGC) ₉	F - GGTTTGGAAGGAGGGAACG R - GATACACGTCAAAGGCGAGC	59.8	7	PET	B	7	277-294
CZ 42	(ATC) ₈	F - CGCGAGAAGTCCATTGAACC R - AGGAGCAAACCAATATGCGG	59.7	5	PET	B	5	337-355
CZ 43	(ATC) ₈	F - AGGCCACGTACTTCCTCTTC R - TGGAGCTATCGTCATCCTGC	60	6	FAM	B	5	107-126

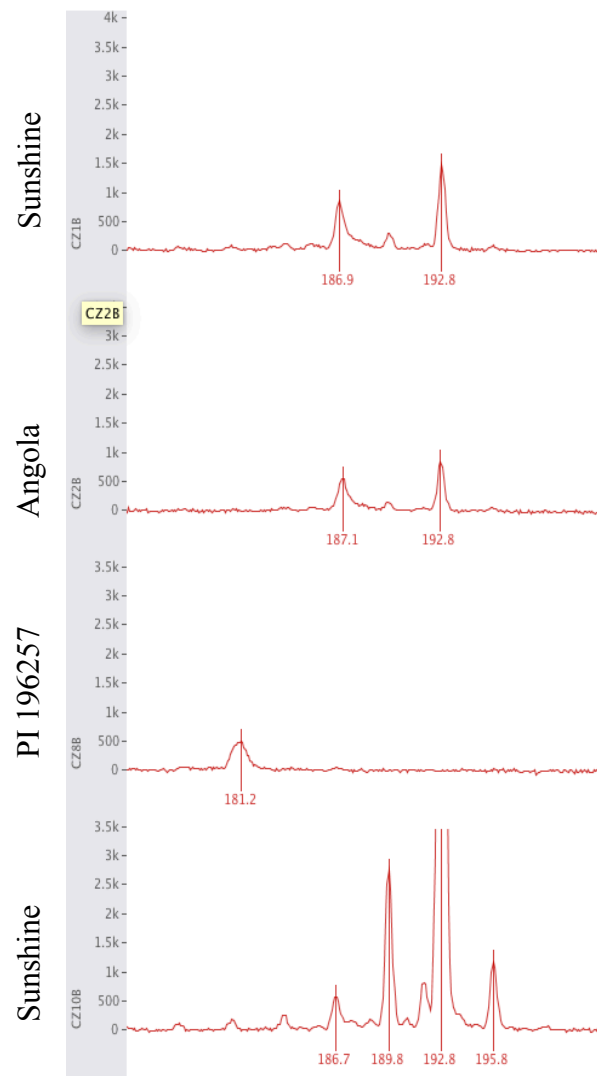
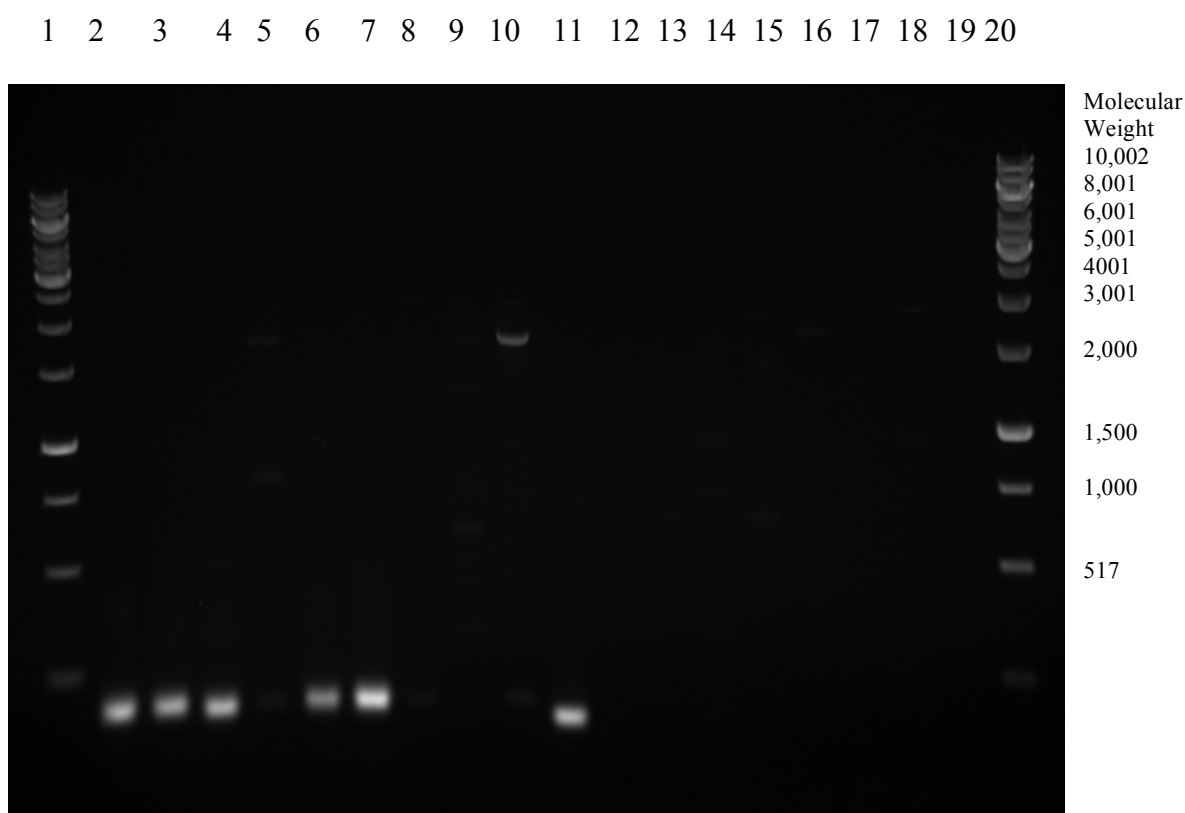


Figure 8. Marker CZ13 peak pattern across four individuals. The first Sunshine individual and Angola share a similar peak pattern. They both appear to be diploid and have peaks at locus number 187 and 193 of the microsatellites. PI 196257 has a lower intensity peak occurring further away from the other alleles that appear to be present; however, it does follow the (AAT)₁₁ motif of CZ13. The second Sunshine individual appears to be tetraploid for this marker; it has four distinct peaks that are about 3 base pairs away from each other, which matches the (AAT)₁₁ motif of CZ13.

A simple agarose gel assay was developed to determine if CZ13 has a presence for only the Sunshine variety (Figure 9). This assay was based on determining if the PCR product of marker CZ13 would show a presence of PCR product for the Sunshine varieties and not amplify for other varieties. Thus, the goal was to see if the genotyping data could be

visualized on an agarose gel. The Sunshine varieties (PI 671958A and B), PI 196257, PI 504803, Capitol, and Angola varieties amplified in one location, resulting in a single band. The remaining 12 individuals did not amplify or had indistinguishable bands. CZ13 amplifies both the Sunshine and the non-sterile varieties, such as PI 196257. Therefore marker CZ13 would not be a good indicator for sterility because it amplifies in non-sterile and sterile varieties of Vetiver.



1 – 1 kbp Ladder
2 – Sunshine
3 – Angola
4 – PI 196257
5 – PI 537061
6 – Sunshine
7 – Capitol

8 – PI 271633
9 – AusTRCF106187
10 – PI 504802
11 – PI 504803
12 – PI 504807
13 – PI 219580
14 – PI 538757

15 – PI 383762
16 – PI 536999A
17 – PI 504808
18 – AusTRCF52213
19 – Negative Control
20 – 1 kbp Ladder

Figure 9. Assay image for marker CZ13, imaged using ethidium bromide and UV light. The assay only compared 18 individuals because biological replicates were removed. Both Sunshines, Capitol, Angola, PI 196257 and PI 504803 amplified strongly for CZ13. Other

individuals, such as PI 537061, AusTRCF106187, PI 504802 were amplifying in more than one region with weak bands.

The fine scale resolution offered by fluorescent genotyping allowed us to identify some individuals as tetraploid (4 distinct amplicons) (Figure 9). This includes *Chrysopogon* individuals PI 383762, PI 536999A, and PI 504803. The distinct tetraploid pattern observed in the chromatograms (Figure 10) are seen throughout the collection and do not appear to be linked to regions and varieties. Below is an example of tetraploids being present in the chromatograms. There is some evidence that tetraploids exist or have been artificially created to increase oil production (Dhawan and Lavania, 1996).

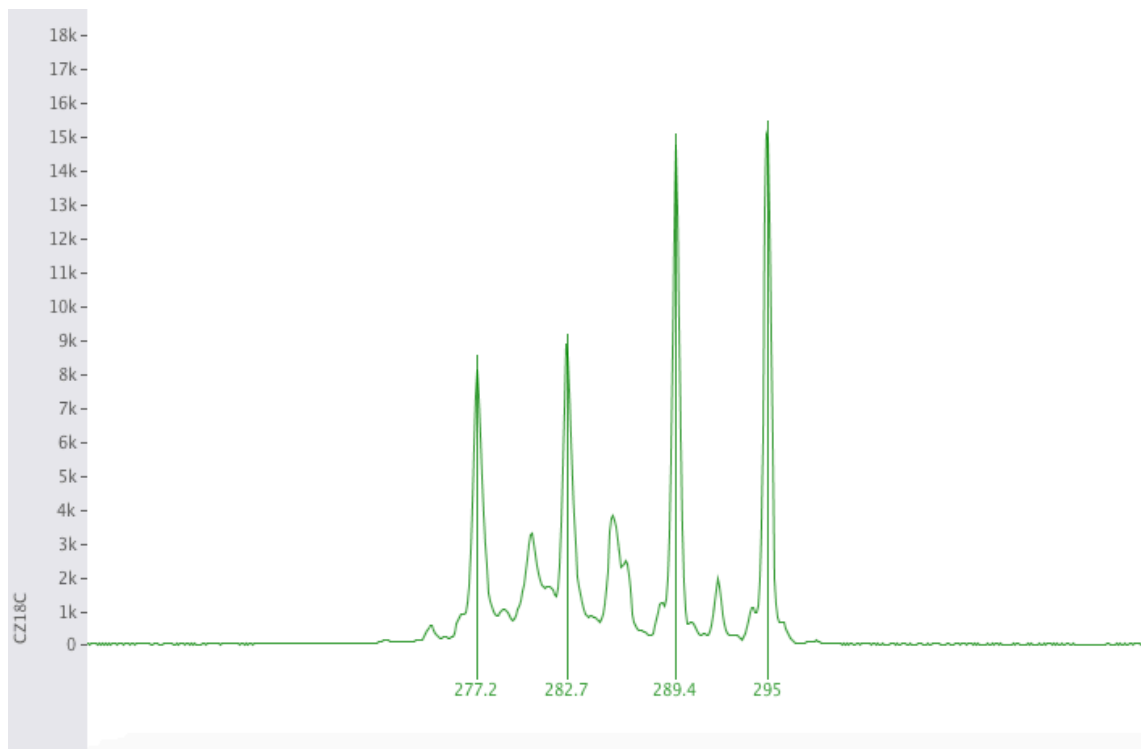


Figure 10. Example of tetraploid individual (PI 504802) using CZ33 labeled with VIC. The distinct four large peaks, with a consistent peak pattern indicates that there are four alleles for one microsatellite for PI 504802. Having four alleles for one trait suggests a tetraploidy occurring.

Conclusions

The microsatellite markers developed in this project were aimed at better identifying varieties of Vetiver to ensure proper monitoring of Vetiver imports. The best method at preventing a Vetiver invasion would be to avoid releasing it in the U.S.; however with Vetiver's agricultural and erosion control advantage, there needs to be a balance between the benefits of Vetiver usage and the damage a potentially invasive species could inflict (Mehta, et al., 2007).

We did not identify a single distinguishing genetic feature for the Sunshine variety. There would be no benefit to developing more microsatellite markers because these markers would amplify in similar regions and have similar results. Further experiments will not include developing more markers. Since a touchdown PCR protocol was used and the microsatellites amplified well, adjusting the PCR protocol would not result in different results. Further investigations of sterility, such as determining pollen viability, should be used to verify if the CZ13 assay is viable. Pollen viability will be able to determine if the variety is producing pollen that can fertilize. If not, the variety would be considered sterile. Determining which individuals are sterile will help determine how accurate the CZ13 assay is.

These results suggest tetraploidy is more common in Vetiver than previously reported. Since tetraploidy is seen across each variant with no pattern, it does not appear to be related to sterility. Clarifying which individuals are tetraploids, using chromosome smashes or flow cytometry, could be useful in better understanding Vetiver genetic diversity.

These markers may also be useful in identifying genetic diversity within the genus

Chrysopogon and within Vetiver. It is important to understand genetic diversity of potentially invasive species because an increase of Vetiver's genetic diversity could make it more adapted to the environment and thus more invasive. Allowing for estimates of genetic diversity in case of an accidental release or in evolutionary studies of the genus.

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